AD_____

Award Number: DAMD17-01-1-0009

TITLE: New Advanced Technology to Improve Prediction and Prevention of Type 1 Diabetes

PRINCIPAL INVESTIGATOR: Massimo Trucco, M.D.

CONTRACTING ORGANIZATION: Children's Hospital of Pittsburgh
Pittsburgh, Pennsylvania 15213

REPORT DATE: November 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	November 2002	Annual (1 Nov 01 - 31 Oct 02)

4. TITLE AND SUBTITLE
New Advanced Technology to Improve Prediction and Prevention of Type 1 Diabetes

5. FUNDING NUMBERS
DAMD17-01-1-0009

6. AUTHOR(S) :
Massimo Trucco, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Children's Hospital of Pittsburgh
Pittsburgh, Pennsylvania 15213

E-Mail: mnt@pitt.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
Type 1 diabetes is considered an autoimmune disease characterized by the presence of lymphocytes in the islets of Langerhans. The majority of infiltrating cells are actually T lymphocytes that are considered responsible for the destruction of the insulin producing β-cells present in the islets. When the number of dead cells reach 85-90% of the originally existing β-cells, the disease presents, frequently with an abrupt and clinically serious onset. Individuals are considered at high risk to develop the disease, based on their genetic susceptibility (as determined by the presence of susceptibility alleles at various HLA loci) and on the presence in the serum of autoantibodies directed against islet specific autoantigens (the most indicative being GAD65, IA-2, and insulin). In individuals genetically at risk for the disease an environmental component is generally considered to be the triggering event. Viruses, and in particular enteroviruses, are among the most influential environmental triggers of the autoimmune reaction that brings about the disease onset. The aim of this program is to determine whom among the Army personnel is at high risk to develop the disease in order to prevent the unexpected onset of the disease that may be associated with tragic consequences.

14. SUBJECT TERMS
Type 1 diabetes, HLA alleles, autoantibodies, T cells, enteroviruses, pyrosequencing

15. NUMBER OF PAGES
22

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

GRANT NO. DAMD17-01-1-0009
ANNUAL TECHNICAL REPORT
Table of Contents

INTRODUCTION

Type 1, or insulin-dependent diabetes mellitus (IDDM), is an autoimmune disease characterized by the presence of infiltrating lymphocytes in the islets of Langerhans. This pathologic scenario is normally defined as "insulitis". The majority of infiltrating cells are actually T lymphocytes that are considered responsible for the destruction of the insulin producing β-cells present in the islets. When the number of dead cells reach 85-90% of the originally existing β-cells, the disease presents, frequently with an abrupt and clinically serious onset.

Diabetes currently affects an estimated 16 million Americans, and about 800,000 new cases are diagnosed each year. New, promising human trials, aimed at preventing the dramatic onset of the disease, are sponsored by the NIH under the organization umbrella known as TrialNet. Individuals considered at high risk to develop the disease, based on their genetic susceptibility (as determined by the presence of susceptibility alleles at various HLA loci) and on the presence in the serum of autoantibodies directed against islet specific autoantigens (the most indicative being GAD65, IA-2, and insulin) are included in these trials.

Since being at-risk for the disease is not alone sufficient for one to eventually become diabetic, an environmental component is generally considered to be the triggering event. Viruses, and in particular enteroviruses, are considered among the most influential environmental triggers of the autoimmune reaction that brings about the disease onset.

To determine whom among the Army personnel is at high risk to develop the disease in order to prevent the unexpected onset of the disease that may be associated with tragic consequences if the individual is performing a particularly difficult task (e.g., the pilot of a bomber plane), we proposed the following major aims for our study:

1) Defining a prophylactic approach against possibly diabetogenic forms of enteroviruses.

2) Setting up an efficient, reliable, and inexpensive molecular approach for typing very large numbers of individuals for the genetic markers associated with susceptibility or resistance to type 1 diabetes.

3) Using a powerful technological tool, the pyrosequencer, to recognize SNPs associated with loci conferring diabetes resistance in mice or associated with complications of diabetes in human.

CVB4-infected Vero cell lysates stimulate *in vitro* CD4+ and CD8+ T cells carrying Vβ7 chain

(Patrizia Luppi, M.D., Division of Immunogenetics, CHP)

Recently, it has been shown that a correlation exists between Coxsackievirus B (CVB) infections and T cell receptor (TCR) gene utilization. Our group has found that αβ+ T lymphocytes infiltrating the pancreatic islets of patients suffering from type 1 diabetes used a restricted set of

rearranged TCR genes (Conrad et al., 1994; Luppi et al., 2000). One hypothesis to explain the presence of a selective activation of T cells *in situ* is the one considering an interaction with a specific antigen -- most probably CVB-derived (19-21) -- that might play an important role in disease pathogenesis. Indeed, evidence showing selective activation of specific T cell subsets in CVB3-induced myocarditis in mice has been recently reported (Huber et al., 2001). In the same study, infection of animals with a non-pathogenic mutant CVB3 stimulated different T cell subsets leading to mild myocarditis.

While CVB3 seems to be cardiotropic, CVB4 is pancreatropic. Experiments aimed at defining the antigen specificity of CD4+ and CD8+ T cells are ongoing in the laboratory. Before testing different proteins of the CVB4 strain for the ability to trigger activation of the specific T cell subsets, we have investigated by flow cytometry which is the phenotype of CD4+ and CD8+ T cells after incubation with CVB4-infected cell lysates. The new data, now presented in Figures 1 and 2, show proliferation and activation of Vβ7+ T cells, within both CD4+ and CD8+ T cell subsets, from different subjects. Conversely, other Vβ families did not show evidence of proliferation, thus confirming the specificity of the CVB dependent activation for only the Vβ7+ T cells.

To evaluate whether CVB-derived antigens might be responsible for the expansion of Vβ7+ T lymphocytes in the pancreata of the diseased patients, PBMC from 3 donors not affected by diabetes were cultured *in vitro* in the presence of lysates of Vero cells infected or non-infected with CVB4. After 24 hours of culture, PBMC were harvested, stained with the different mAb and analyzed for expression of Vβ7+ chain within CD4+ and CD8+ subset of lymphocytes by flow cytometry. We choose CVB4 for our *in vitro* challenge as signs of CVB4 infections were found in our diabetic patients by immunochemistry.

The results of our experiments demonstrated that the percentage of CD4+ and CD8+ T lymphocytes bearing TCR Vβ7 chain increases in all the donors after 24 hours of stimulation with CVB-infected Vero cell lysates, as compared with the values for these cell populations measured in the PBMC of the same individuals cultured with lysates of non-infected Vero cells (Fig. 1). The expansion of Vβ7+ T cells was also associated with expression of the leukocyte antigen CD69, a marker of early cell activation (Fig. 2). When PBMC from the different donors were exposed to Vero lysates, the percentage of CD4+/Vβ7+ lymphocytes was 28.3±9.7% as compared to the percentage measured in the cultures with lysates of Vero cells not exposed to the virus (2.7±0.7%). Expansion of Vβ7+ T cells was also detected within the CD8+ T cells compartment after incubation with CVB4 lysates, with an average value of 20.9±9.1%. The percentage of CD8+/Vβ7+ T cells cultured in the presence of uninfected cell lysates was 4.0±0.7%. Conversely, the analysis of the percentage of CD4+ and CD8+ T cells carrying, for example, the Vβ8 chain after culture with CVB4-infected Vero cell lysates (4.4±1.6% and 5.0±1.5%, respectively) did not show any significant changes as compared with the level of the same family in the PBMC exposed to the uninfected lysates (4.1±0.5% and 4.0±1.3%, respectively). When we looked at the expression of the activation marker CD69, within the CD4+ and CD8+ T cell compartments of the different donors, when incubated in the presence of CVB4 infected or non-infected Vero cell lysates, we found that there was an up-regulation of this

marker in Vβ7+ T cells after culture with the viral lysates (21.6±8.6% and 13±3.1%, respectively) as compared with the values measured in the absence of viral stimulation (0.9±0.6% and 0.8±0.6%, respectively). Figure 1 and 2 show an example of flow cytometry analysis performed on PBMC from a donor who showed the average level of TCR Vβ7 expansion in both the CD4+ and CD8+ lymphocyte subsets after culture with the viral lysates.

Isolation of PBMC from non-diabetic donors. PBMC were obtained by Ficoll-Hypaque centrifugation (1-Step Lymphoprep, Accurate Chemical & Scientific Corporation, Westbury, NY) of 20ml heparinized blood obtained from normal donors. As described below, three x 10^6 isolated PBMC were used for total RNA extraction and used for cDNA synthesis and TCR repertoire analysis, while four x 10^6 PBMC were used in *in vitro* experiments aimed at analyzing the phenotype of T lymphocytes activated by CVB4-infected Vero cell lysates, as described below.

In vitro PBMC stimulation with CVB4-infected Vero cell lysates. PBMC (4 x 10^6) isolated from three different donors not affected by the disease were seeded in a volume of 3ml of RPMI-1640 medium (Gibco BRL) supplemented with 10% human AB serum (Sigma Chemical, St. Louis, MO), into a 0.2μm ventilation-capped 25cm2 tissue polystyrene culture flasks and exposed to CVB4-infected Vero cell lysates (Virion Inc., Ruschlikon, Switzerland). These lysates do not have any infectious potential. Before addition to the culture, the lyophilized cell lysate was reconstituted with 1 ml distilled water, centrifuged at 13,000 rpm for 5 minutes and resuspended in 1 ml of culture medium. This procedure appears to eliminate the toxic effects of the lysate while immunogenic effects are still maintained (Virology 274:56, 2000). PBMC were cultured at 37°C in 5% CO_2 for 24 hours. After incubation, cells are pelleted and resuspended in FACS media (phosphate-buffered saline with 0.5% bovine serum albumin and 0.1% sodium azide; Sigma Chemical Co.), aliquoted and stained with saturating concentration of the following anti-human monoclonal antibodies (mAb): fluorescein isothiocyanate (FITC)-conjugated mAb to various human Vβ chains (Immunotech, Marseilles, France); phycoerythrin (PE)-conjugated mAb to CD69 (Immunotech); peridin chlorophyll protein (PerCp)-conjugated mAb to CD8 (Becton Dickinson, San Jose, CA); and allophycocianin (APC)-conjugated mAb to CD4 (Becton Dickinson). The negative-control panels were comprised of mixtures of isotype negative controls diluted to an equivalent immunoglobulin concentration. All the samples were stained in the dark for 15 minutes, washed with 1 ml FACS media and centrifugated at 2,000 rpm for 5 minutes. The cell pellet was then resuspended in 1% paraformaldheyde (PFA) and stored at 4°C until run on a flow cytometer within 4 hours.

Flow cytometry and data analysis. Stained PBMC from the different donors were analyzed using a Becton Dickinson (San Jose, CA) FACS-Calibur machine calibrated with CALIBRITETM beads. At least 30,000 events were collected for each antibody combination, and the data saved for later analysis on a CELLQuest software (Becton Dickinson). The percentage of Vβ7+ T cells present in PBMC after culture with either CVB4-infected or non-infected Vero cell lysate was calculated by setting gates around the CD4+ and the CD8+ elements based on dot-plot graphs of side-light-scatter (SSC) versus the positive cells for each antibody specificity.

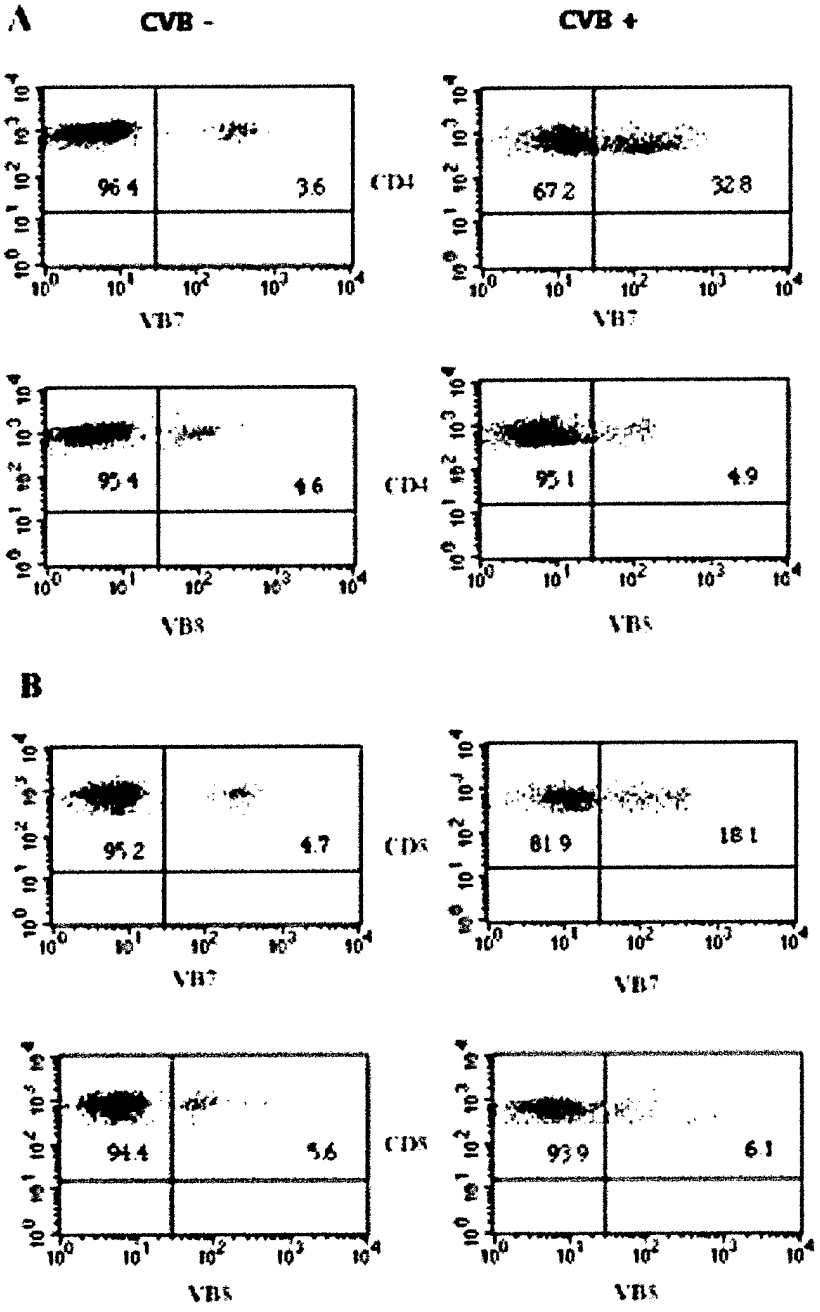


Figure 1. Flow cytometry analysis of Vβ7+ T cells in CD4+ and CD8+ lymphocyte subsets in the presence of infected (CVB+) and non-infected (CVB-) Vero cell lysates. Shown is a representative example of Vβ7+ expansion in CD4+ (**A**) and CD8+ (**B**) T cell subsets after *in vitro* stimulation with CVB4-infected Vero cell lysates. After culture, PBMC were harvested, stained with a panel of mAb to different Vβ chains and to CD8 and CD4 antigens and processed for flow cytometry analysis. In **A**, it is represented the percentage of Vβ7+ cells in CD4+ cells after culture with either uninfected (3.6%) or CVB4-infected Vero cell lysates (32.8%). Frequency of Vβ8+ T cells is also shown as an example of the other TCR Vβ chains that did not show any change between the two culture conditions. In **B**, it is represented the percentage of Vβ7+ cells in CD8+ cells after culture with either uninfected (4.7%) or CVB4-infected Vero cell lysate (18.1%). Frequency of Vβ8+ T cells is also shown as an example of the other TCR Vβ chains that did not show any change between the two culture conditions.

Within the CD4+ and the CD8+ gate, Vβ7+ positive elements were further analyzed for the expression of the early activation marker CD69. Following the same protocol, other Vβ families besides Vβ7 were also tested as controls. For each population, an isotype-control mAb was used to define the placement of the negative marker. Fluorescence was measured using a log10 scale. The results are given as proportion (percentage – SD) of CD4+ or CD8+ T cells expressing a specific Vβ family and CD69.

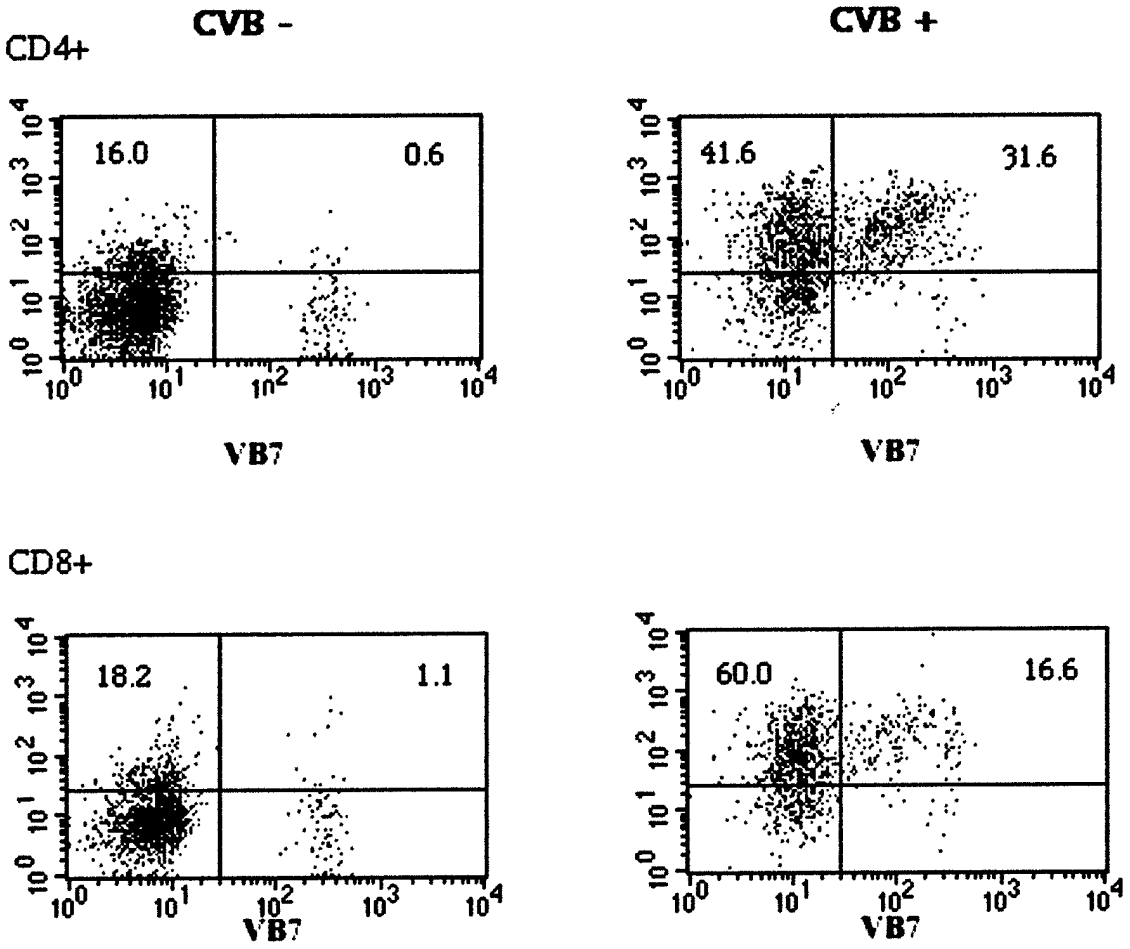


Figure 2. CVB4-stimulated Vβ7+ T cells express the early activation marker CD69. Shown is a representative example of flow cytometry analysis of CD69 expression on Vβ7+ lymphocytes within the CD4+ (top panel) and CD8+ (bottom panel) T cell subset after 24 hours culture with either uninfected (CVB-) or CVB4-infected Vero cell lysates (CVB+). The proportion of Vβ7+ T cells expressing the activation marker CD69 is expressed as a percentage of Vβ7+ T cells within the CD4+ and CD8+ gated population of lymphocytes. An increase in the percentage of Vβ7+ also expressing the activation marker CD69 within the CD4+ T cells (top panel) and the CD8+ T cells (bottom panel) was detected.

Pyrosequencing sheds light on HLA genotyping and sets the basis for SNP analysis of mouse and human genomes.

(Angela Alexander, Ph.D., Steve Ringquist, Ph.D., Division of Immunogenetics, CHP)

HLA Genotyping

Pyrosequencing was originally designed for expressed sequence tag (EST) sequencing in which short (roughly 10 nucleotide) stretches of DNA are analyzed. The pyrosequencing method, reviewed recently (Fakhrai-Rad, 2002; Ronaghi, 2001), exploits a multi-enzyme process combining the activities of DNA polymerase, ATP sulfurylase, apyrase, and luciferase in the conversion of inorganic pyrophosphate (a product normally generated upon incorporation of a nucleotide into a nascent DNA chain) into light. Generation of light signals incorporation of a particular nucleotide. Pyrosequencing is performed by reiteration of steps in which an individual dNTP is added one at a time and tested for incorporation into the nascent nucleotide chain, similar to a process of probing but by single nucleotide hybridization, well suited to analysis of polymorphic DNA. Pyrosequencing has been readily able to read DNA sequences of up to 70 nucleotides (Alexander et al., 2002; Ringquist et al., 2002), and sequences as long as 150 residues have been reported (Gharizadeh et al., 2002), as well as the shorter lengths required for SNP typing. An important advantage of pyrosequencing over other sequencing technologies is that, because nucleotides are tested individually, the methodology allows the resolution of allelic combinations which are difficult to distinguish by conventional sequencing approaches (Ringquist et al., 2002). Moreover, development of HLA allele specific pyrosequencing primers has greatly improved the method's resolution during HLA genotyping (Alexander et al., 2002; Ringquist et al., 2002). Similarly, multiplex pyrosequence analysis strategies have been developed in which 2 or more sequencing primers can be used concurrently during pyrosequencing (Pourmand et al., 2002).

Sequencing of alleles of the highly polymorphic class II HLA-DR and DQ gene families was performed by pyrosequencing. Accurate genotyping was achieved using DNA from HLA homozygous cell lines as well as from blood samples obtained from adult volunteers. Pyrosequence-ready DNA was routinely prepared from donors whose blood had been stored either frozen or as dried blood spots. Pyrosequence-Based Typing was optimized for identifying alleles of the expressed HLA-DRB genes 1, 3, 4, and 5 as well as HLA-DQB1 as reported previously (Ringquist et al., 2002). The procedure should be applicable to other HLA loci, including the class I genes HLA-A and B which, along with HLA-DRB and DQB, are crucial for histocompatibility matching of tissue antigens during transplantation as well as risk evaluation of an individual's likelihood of developing type 1 diabetes mellitus and a variety of other autoimmune disease. Pyrosequencing and PCR primers could be designed to enable specific sequencing of HLA loci of interest even in a background of other amplified, closely related sequences such as the HLA-DRB pseudogenes 6, 7, 8, and 9 as well as the DQB2 pseudogene. All of the polymorphic residues, embedded in exon 2, of HLA-DRB and DQB class II genes were identified within a few pyrosequencing reactions obtained by 50 to 70 nucleotide read lengths. Heterozygous allelic combinations of HLA genes were analyzed and compared successfully to genotyping of alleles by sequence-specific oligonucleotide probe hybridization as well as allele

specific PCR protocols. Pyrosequence-Based Typing is compatible with genotyping of allelic combinations expected from heterozygous individuals, resulting in nucleotide resolution of the highly polymorphic HLA system. Using a single pyrosequence instrument, complete typing of HLA-DRB class II genes can be performed daily on hundreds of individuals for high resolution histocompatibility genotyping studies.

Accurate HLA genotyping is essential for matching of tissue during bone marrow as well as solid organ transplantation (Hsia et al., 1993; Tong et al., 1993). Moreover, analysis of genomic DNA obtained from families plagued by high occurrence of autoimmune disease have provided critical information linking the risk of developing autoimmune disorders to certain HLA alleles. For example, nucleotide polymorphisms at HLA class II loci have correlated with susceptibility to a variety of autoimmune diseases, such as type 1 (insulin dependent) diabetes (Friday et al., 1999), celiac disease (Sollid, 2002), and rheumatoid arthritis (Peakman, 1999). A wide body of evidence has correlated specific polymorphisms of the HLA-DRB and DQB1 loci, such as DR3 or DR4 along with inheritance of non-Asp-57 residue in the DQB1 gene product, as being the strongest indicators of susceptibility for type 1 diabetes (Trucco, 1992). Meanwhile, the HLA DR3-DQ2 haplotype has been linked with celiac disease (Sollid, 2002), and inheritance of the HLA-DRB1*04, *01, *14, or *10 subgroups have been associated with markedly increased risk of developing rheumatoid arthritis (Peakman, 1999).

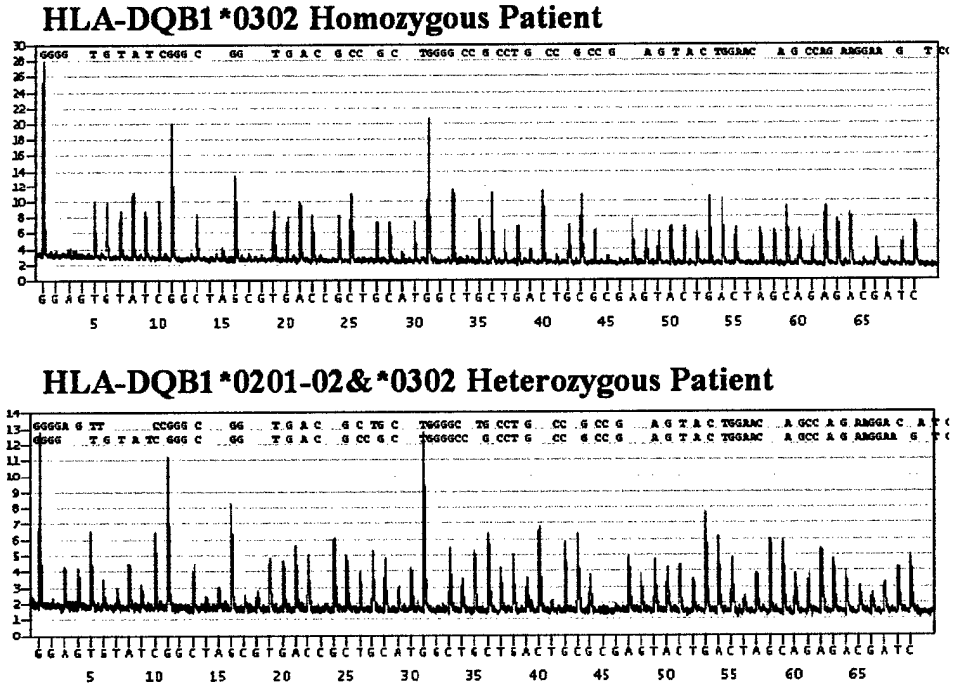


Figure 3. Pyrograms of HLA-DQB1 alleles prepared from peripheral blood mononuclear cells extracted genomic DNA samples from volunteer donors. Pyrograms illustrate sequence data from a homozygous *0302 individual (top) and a heterozygous *201-02&*0302 individual (bottom). Nucleotide sequences are indicated for *0302 in top panel and for *0201-02 (upper sequence) and *0302 (lower sequence) in bottom panel. Read lengths were at least 70 nucleotides.

Development of rapid and cost effective strategies for screening of the highly polymorphic HLA loci for alleles associated with autoimmune disease is important for risk evaluation of genetically susceptible individuals. The recently described method of Pyrosequence-Based Typing (PSBT) (Ringquist et al., 2002) provided high resolution genotyping of alleles of the HLA-DQB1 locus, unambiguously identifying HLA-DQB1 alleles obtained from PCR amplified samples of genomic DNA from cell lines as well as DNA isolated from human donors (Figure 3). Pyrosequencing experiments indicated that analysis of 70 nucleotide regions of HLA-DQB1 exon 2 from donor blood samples provided high resolution sequencing data for homozygous as well as heterozyous genotypes. Comparison of results from homozygous HLA-DQB1*0302 and heterozygous HLA-DQB1*0201-02&*0302 genotypes indicated that the pyrosequence peak heights closely followed the expected sequences from these individuals (Figure 3). PSBT was also able to distinguish the pyrosequencing signals from the HLA-DQB1 alleles *0201-02 and *0302 from the heterozygous donor (Figure 3, bottom panel). For example, pyrosequence nucleotide dispensations G4, T26, T34, C65, and A67 were specific for the *0201-02 allele while dispensations G6, T7, A8, T9, and G66 were specific for allele *0302.

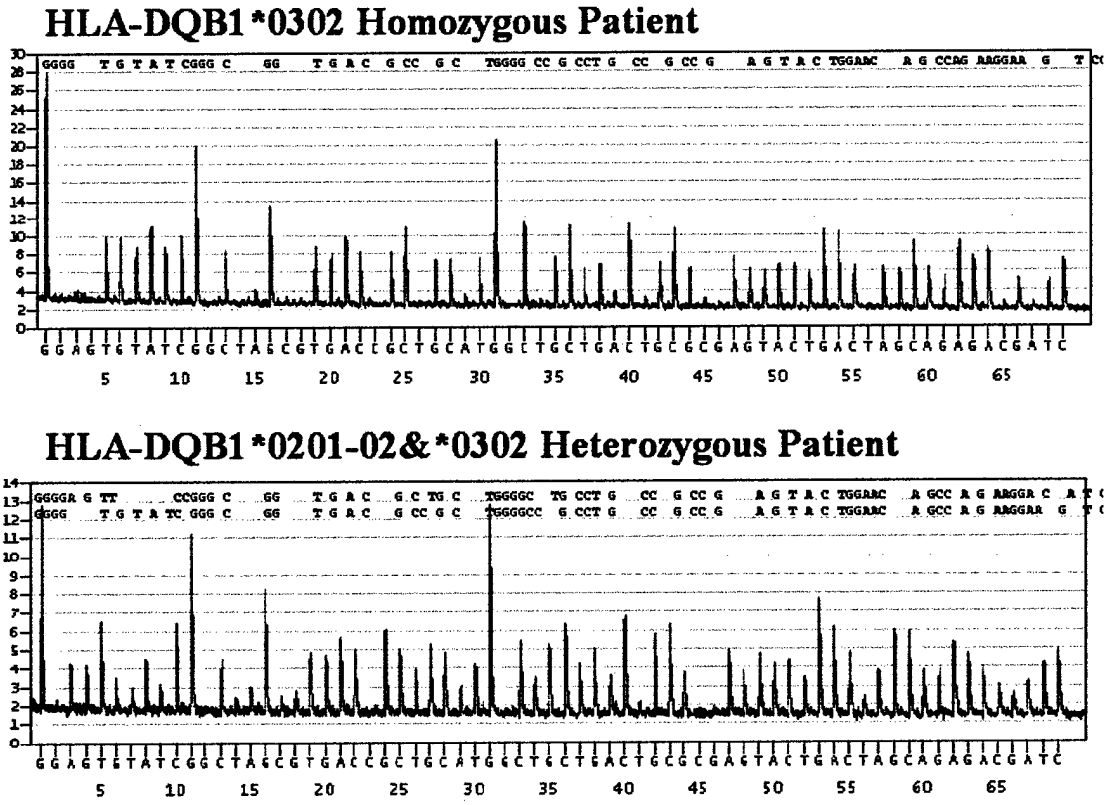


Figure 4. Pyrosequence of an 18 nucleotide region of exon 2 of the HLA-DQB1 gene for an HLA-DQB1*03011&*0302 heterozygous individual. Pyrosequencing of amplified genomic DNA resulted in pryogram peak intensities corresponding to the expected sequence for the combined *03011 and *0302 alleles. Polymorphisms associated with aspartate (*03011) or alanine (*0302) at codon 57 are indicated at dispensations G38, A39, and C40 (blue). Background signal observed at nucleotide dispensations T34, T41, C45, and G46 are as expected for the DQB1 *03011&*0302 genotype; the dispensation order having been optimized to allow sequencing of all combinations of DQB1 alleles in this region.

PSBT succeeded in determining the identity of allele polymorphisms encoding amino acid substitutions at specific, disease-associated codons, such as the presence of a non-Asp residue at DQB1 codon 57 (Figure 4), as well as in obtaining high resolution sequence data leading to unequivocal allelic genotyping that, along with haplotype information, are known to improve accuracy when evaluating an individual's risk for developing HLA-linked autoimmune diseases (Trucco, 1992). Pyrograms obtained for genomic DNA from a donor whose HLA genotype is DQB1*03011&*0302 clearly illustrated the presence of codons for Asp-57 (DQB1*03011) and Ala-57 (DQB1*0302) which, when present along with the DR3 or DR4 alleles, indicate a greatly increased predisposition to developing autoimmune-associated type 1 diabetes mellitus (Trucco, 1992). Alleles *03011 and *0302 specific nucleotide dispensations are indicated in Figure 4 at positions G38, A39, C40 for the aspartate and alanine codons. Moreover when applied to HLA-DRB and the class I genes HLA-A and B, PSBT will be applicable to high resolution typing of HLA alleles required during successful kidney and bone marrow transplantation. PSBT can provide high resolution SNP analysis for monitoring inheritance of specific amino acid substitutions as well as identifying alleles of inherited polymorphic DNA for genotyping over large exon length regions.

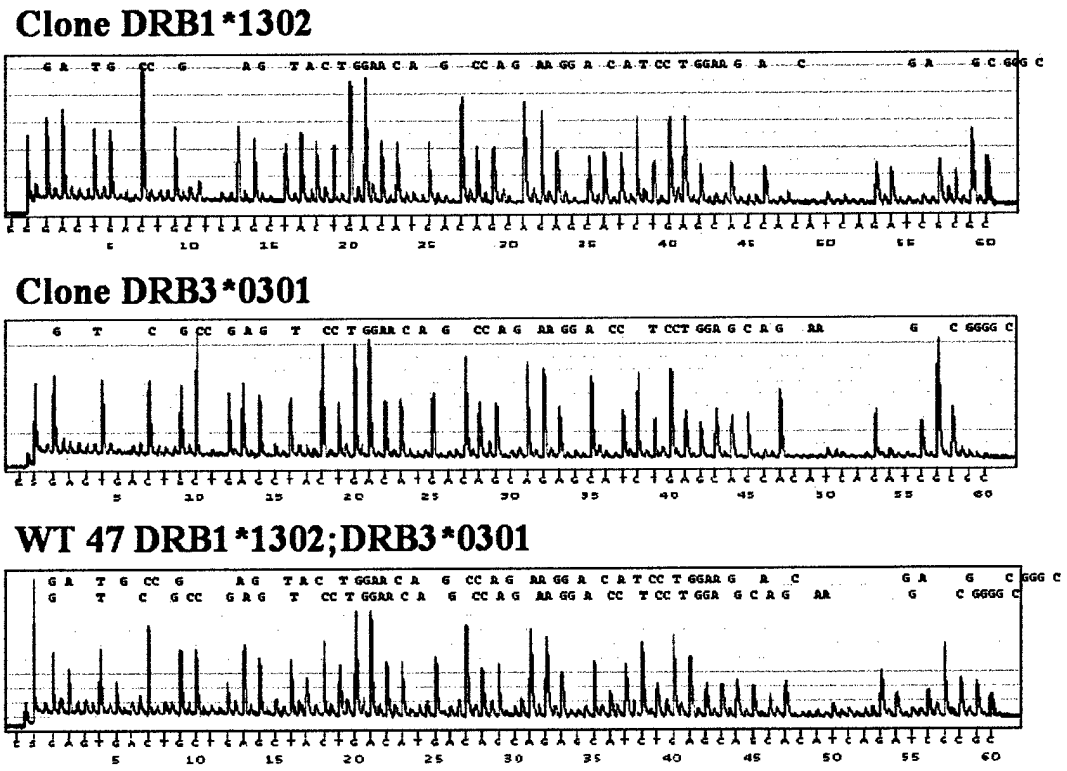


Figure 5. Pyrograms of cloned HLA-DRB genes DRB1*1302 and DRB3*0301 and the cell line WT47 HLA-DRB1*1302;DRB3*0301 genomic DNA. Samples were prepared for pyrosequencing from cloned DNA (top and middle panels) or from genomic DNA purified from the HLA containing cell line WT47 (bottom panel). The nucleotide sequence for the HLA-DRB alleles for each sample are indicated. Read lengths were at least 50 nucleotides.

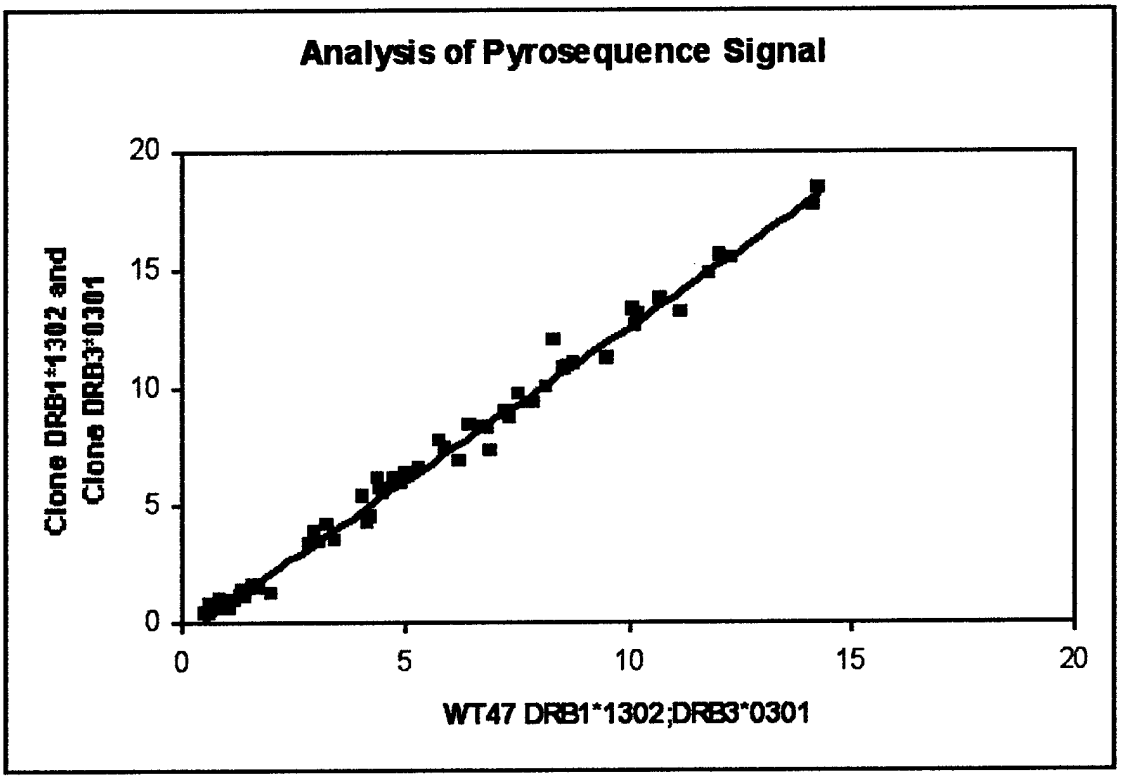


Figure 6. Comparison of pyrosequencing signal from the peak heights shown in the pyrograms from Figure 5. Peak heights for each nucleotide dispensation were compared. The peak heights from the cell line WT47 DRB1*1302;DRB3*0301 are indicated in the x-axis while the weight average of the peak heights observed from clones DRB1*1302 and DRB3*0301 are indicated in the y-axis. Linear regression analysis indicated an r2 value of 0.99.

We have expanded on our previous work, developing PBST for the identification of HLA-DQB1 alleles in clinical samples (Ringquist et al., 2002), while simultaneously tackling the genotyping of the complex multi-loci HLA-DRB system (Alexander et al., 2002). As illustrated in Figure 5, HLA-DRB genes have been successfully analyzed by pyrosequencing. Comparison of pyrograms obtained from cloned DNA and cell line genomic DNA has indicated, at least for HLA-DRB alleles DRB1*1302 and DRB3*0301, that genomic DNA can be successful analyzed. Analysis of the pyrograms indicated that DRB1*1302 specific nucleotide dispensations occurred at events A2, G5, A17, A36, A41, C46, A54, G59, and C60 while DRB3*0301 associated events were at dispensations C10, G12, C43, G45, A47, and C56. The plot, illustrated in Figure 6, also indicated that HLA-DRB1*1302 and DRB3*0301 whether obtained from cloned or from genomic DNA resulted in identical pyrosequencing signal intensities. Plotted are peak height data from each nucleotide dispensation observed from pyrosequencing of the WT47 cell line (DRB1*1302;DRB3*0301) on the x-axis against the weight average of the peak height intensities from clones DRB1*1302 and DRB3*0301 on the y-axis (Figure 6). Linear regression analysis indicated a regression coefficient of roughly 0.99. PSBT of HLA-DRB, while matching the

expected sequence for these alleles, yielded readily interpreted pyrosequence data whether originating from cloned stocks or from genomic DNA (Figures 5 and 6).

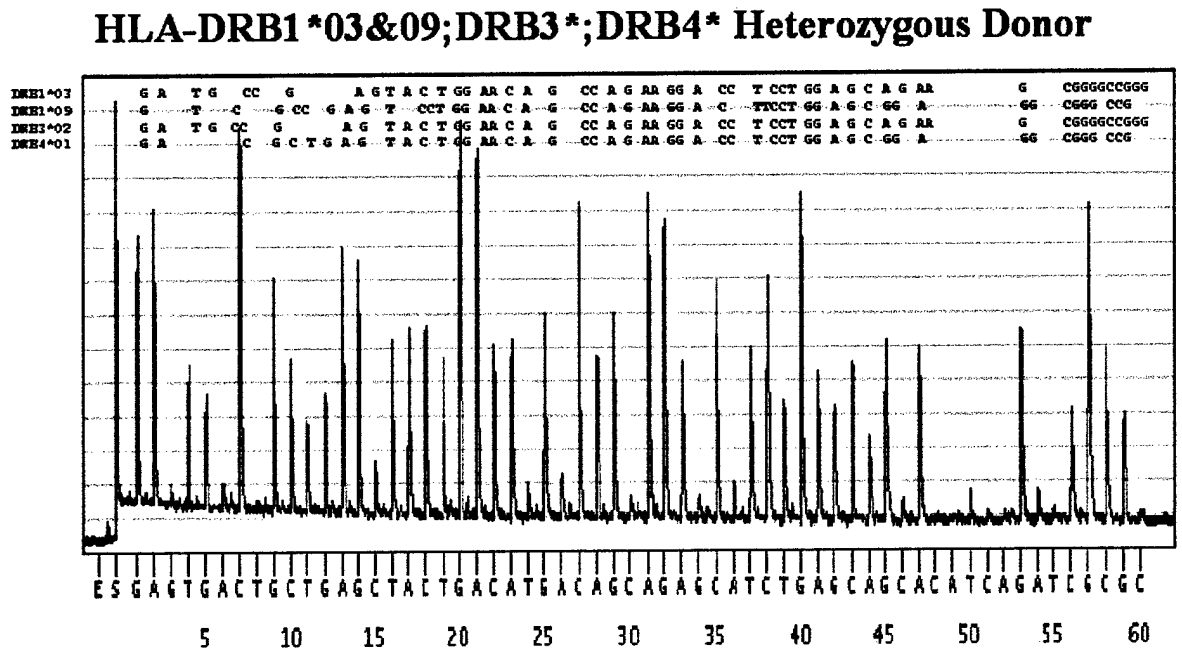


Figure 7. Pyrosequence of exon 2 of HLA-DRB from a DRB1*03&09;DRB3*;DRB4* heterozygous donor. Samples were prepared from whole blood obtained from our donor population. The likely DNA sequence for the allelic combination is indicated. Read lengths were at least 50 nucleotides.

PSBT of HLA-DRB represented the added challenge of typing polymorphic DNA from as many as 4 expressed loci whose knowledge is essential for successful matching of recipient and donor tissue during transplantation as well as during evaluation of susceptibility to autoimmune disease. As illustrated in Figure 7, pyrosequencing was successfully obtained using genomic DNA from blood. This material was typed in parallel using commercially available HLA typing SSOP kits (Dynal Inc.) in order to confirm its identity once examined by PSBT. Both methods for HLA gneotyping analysis were consistent with HLA-DRB1*03&09;DRB3*;DRB4* for this sample. The sequence of the most likely 4 DRB alleles, consistent with the SSOP typing data, are matched to the pyrogram in Figure 7. Moreover, the HLA-DRB and DQB system consists not only of multiple expressed loci but a variety of pseudogenes as well. Analysis of genomic DNA from cell lines (Figure 5) and our donors (Figures 3,4, and 7) indicated that pseudogene sequences were not in evidence during pyrosequencing. Indicating that specific PCR amplification and careful choice of HLA specific pyrosequencing primers were sufficient for generation of high quality PSBT data.

We are currently pursuing cloning of DRB and DQB alleles from our donor population. As illustrated in Figures 5 and 6, we believe that comparison of PSBT data obtained from genomic DNA and that obtained from the isolated cloned alleles will provide a necessary test of the validity of our HLA genotyping system. Additional experiments are focused on using DRB group specific primers during pyrosequencing. Computer analysis of the HLA-DRB and DQB sequences, obtained from the IMGT/HLA database (http://www.ebi.ac.uk/imgt/hla/), have been performed in order to identify suitable sequences for priming pyrosequencing. Group specific primers, once experimentally validated, will allow isolation of specific DRB signals from a population of amplified HLA genes and will provide the flexibility of identifying only those sequences during the PSBT without the added burden of multiple PCR preparations. Primers are being tested that are specific for DRB group alleles DRB1*03, *09 as well as DRB3* and DRB*4. Successful completion of this phase will allow the generation of PSBT data from these alleles individually, greatly improving our ability to accurately identify the presence of a particular HLA genotype.

Furthermore, the use of Pyrosequencing can be successfully applied to improve sample throughput as well as allele resolution for HLA genotyping. DNA extracted from whole blood samples and dried blood spots was typed by pyrosequencing and compared to results using SSP (sequence specific primer), SSOP (sequence specific oligonucleotide probe) and reverse blot technologies. Pyrosequencing was able to resolve previously ambiguous allele combinations in a short time compared to standard techniques which normally require SSOP group specific DRB1 amplifications (Alexander et al. 2002). Pyrosequencing, which was designed to study short (approximately 10 nucleotide) sequence polymorphisms like expressed sequence tags (EST) or single nucleotide polymorphisms (SNP), once adapted to resolve longer sequences (70-100 bases), represents a valuable tool useful for increasing sample throughput and specificity of results for HLA typing, and is easily applicable to the analysis of other genetic loci.

The standard techniques (SSOP, SSP and reverse blot) used for intermediate resolution level HLA genotyping frequently results in detection of ambiguous groups of alleles due to sequence homology between many alleles at the locations of probe or primer hybridization (Figure 8). This means that approximately 25% of DRB1 (or even higher percentages of other loci) typed samples need further testing with allele specific amplification and a repeat of the hybridization process. By employing pyrosequencing at this point, the > 6 hour hybridization through detection steps can be eliminated and replaced by one hour of pyrosequencing. Thus reducing by at least 80% the amount of time needed for resolving the ambiguous samples.

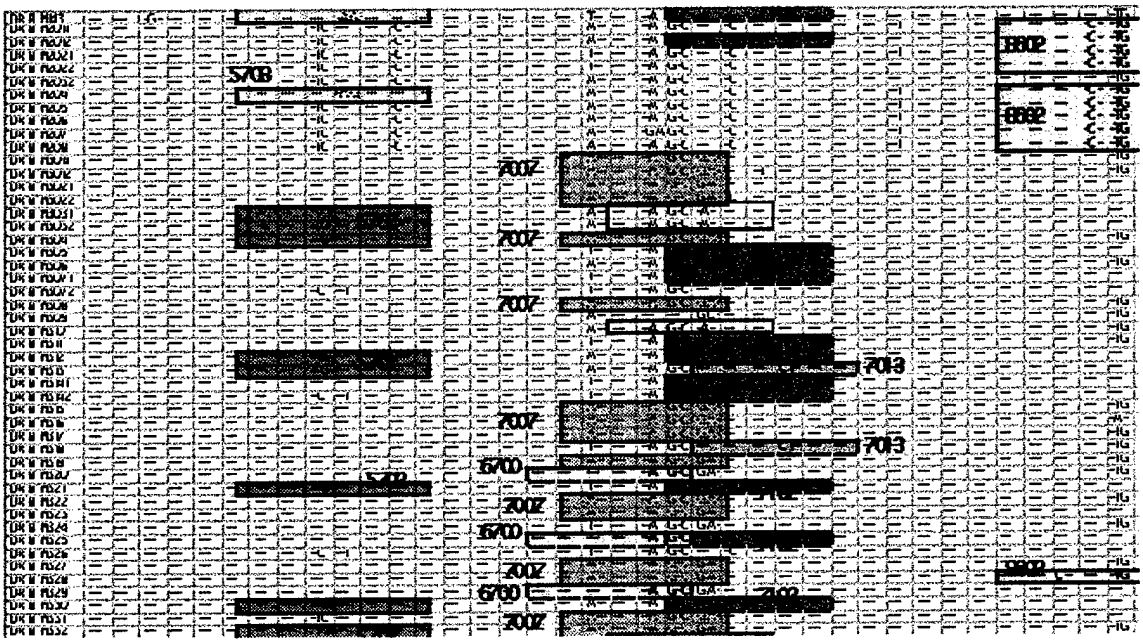


Figure 8. SSOP probe hybridizations cover regions of sequence homology over a wide range of alleles.

Sequencing through informative regions that are not covered by hybridization probes or primers allow further allele identification (Figure 9). Additionally, heterozygous samples can be easily confirmed by differences in signal intensity when nucleotides are detected from only one allele (Figure 10).

Example 1:
Standard SSOP results for sample DG804 are ambiguous. In the presence of DRB3 in the amplicon, the existence of 03012, 0311 0314 and 0317 cannot be confirmed because of sequence homology with DRB3 alleles at the location of probe hybridization.

Total possible DRB1 alleles are:
03012, 0311, 0314, 0315, 0317, 13011, 13012, 13021, 13022, 1309, 1316, 1322, 1323, 1324, 1327, 1328, 1331, 1334, 1335, 1336, 1339, 1340, 1341.

Standard repeated amplification and hybridization can reduce the sample complexity to: 13011, 13012, 1316, 132, 1331, 1335, 1340.

Pyrosequencing with three primers can reduce the sample complexity to:
13011, 1335, 1340. Note that by adding one additional primer to the set, or adjusting primer locations, allele-level resolution can be achieved (i.e. 13011).

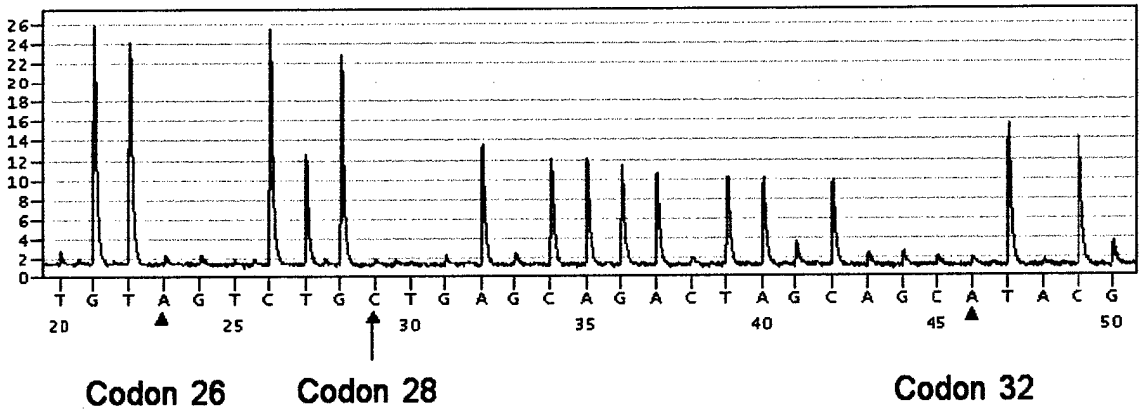


Figure 9. Presence or absence of signal at specific locations (not probed by SSOP) can eliminate/confirm existence specific alleles or groups of alleles.

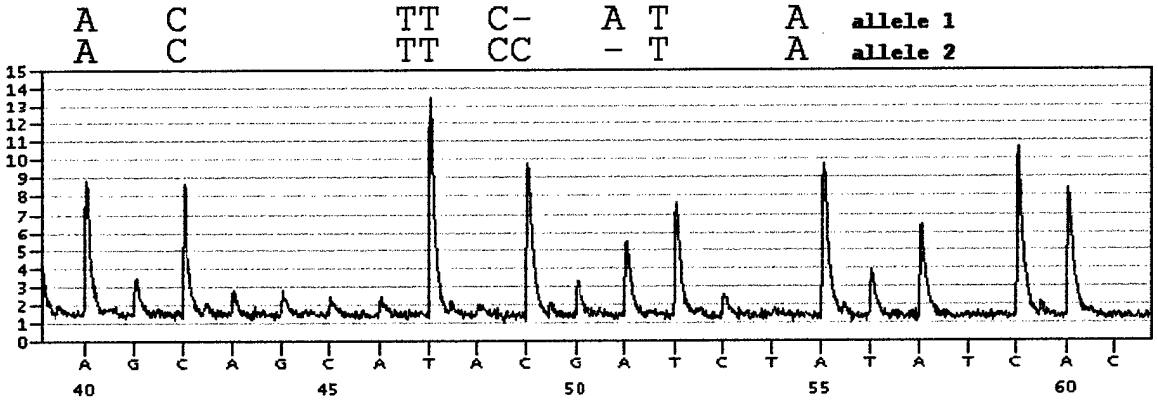


Figure 10. Heterozygous regions are easily detected.

However, depending on the allele combination, pyrosequencing alone is not able to completely resolve ambiguities unless additional steps are taken to separate the individual DNA strands in order to analyze the haplotype inherited from each parent (HaploPrep, Qiagen). Sequence analysis performed after this separation will allow unquestionable determination of alleles present in each sample by detecting each polymorphism as it occurs along a single strand of DNA (rather than a mixture of both inherited strands).

Example 2:
Sample DG806 Results AFTER groups specific SSOP:
1102, 11041, 11042, 1113, 13011, 13022, 1311, 1316, 1328, 1335, 1340, 1342

Results after Pyrosequencing:
1102, 11041, 1116, 13011, 1311, 1342

Potential Results after Pyrosequencing of HaploPrep treated DNA:
1102, 13011

Pyrosequencing was initially used to identify alleles associated with the highly polymorphic exon 2 region of the HLA-DRB1 and the HLA-DRB expressed genes 1, 3, 4, and 5. The ability to generate high resolution sequence data, and throughput of hundreds of samples per day, make PSBT an important method for identification of known genetic markers. In studies of the genetic association of certain HLA alleles with the likelihood of developing autoimmune disease, as well as during clinical genotyping of patients and donors for histocompatibility matching during transplantation, PSBT is a crucial tool for rapid and accurate identification of HLA genotype.

SNP Analysis

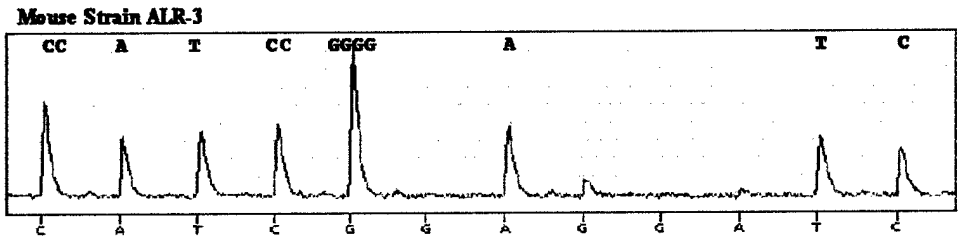
Pyrosequence-Based genotyping of Single Nucleotide Polymorphisms (SNPs) has been performed on mouse genomic DNA (Figure 11). SNP detection is conducted in a manner similar to analysis of polymorphic DNA but usually is focused on identification of a specific nucleotide residue. In the example shown in the figure, mouse strains ALR-3 and NOD1 were compared for the presence of an SNP on chromosome 17. This SNP has been previously reported in the mouse physical map (Gregory et al., 2002) as well as in the comprehensive mouse genome database (http://mouse.ensembl.org) maintained by the European Bioinformatics Institute. As indicated in the figure, our ALR-3 and NOD1 stains exhibited different genotypes when this residue was examined. Allele specific pyrosequencing signals occurred at nucleotide dispensations G5 indicative of the presence of the C residue containing allele and at dispensations G8 and A10 for the A residue containing allele. The ALR-3 strain is homozygous (C/C) while our NOD1 population is heterozygous (C/A) at this position. Judicious programming of the order of nucleotide dispensations can be used to generate convincing pyrosequencing data via the application of "out-of-phase" sequencing. We are currently extending our SNP scan for specific, roughly 20 cM, regions of mouse chromosomes 3, 8, and 17 which have been previously associated with altered susceptibility to autoimmune-mediated diabetes mellitus. Moreover, are using the experience gained from SNP analysis in our mouse system to develop a global

pyrosequence based SNP analysis in humans. In collaboration with Dr. Richard Spielman at the University of Pennsylvania were are beginning to design PCR and pyrosequencing primers for identification of SNPs associated with enhanced risk for developing severe diabetic complications, such as blindness and renal failure.

A.

Allele-(C): CCATC CGGGGATC
Allele-(A): CCATC AGGGGATC

B.



C.

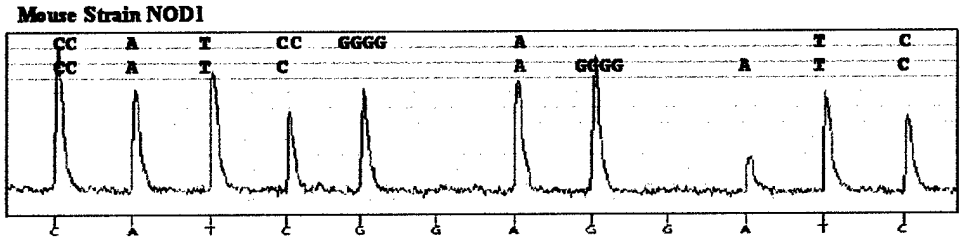


Figure 11. Pyrosequence-Based genotyping of a C/A polymorphism on mouse chromosome 17. The sequences flanking the C/A polymorphism are shown in panel A and the polymorphic residues are indicted in blue. The panels B and C illustrate pyrosequence results from mouse strains ALR-3 and NOD, respectively. The alleles present in each strain are indicated and the polymorphic residues are shown in blue. Pyrosequence ready DNA was prepared from purified high molecular weight genomic DNA and was PCR amplified using primers TCTACGTGTTGGACAGCAGC and CTGGGCGCTAGTATTCCAAG. The latter primer was modified to contain a 5' Biotin residue. The pyrosequencing primer was GCACCATGTTAG.

REFERENCES

Alexander A, Nichol L, Ringquist S, Styche A, Rudert W, Trucco M. Pyrosequencing sheds light on HLA genotyping. Hum Immunol. 2002: **63**(10 Suppl): S95.

Conrad B, Weidmann E, Trucco G, Rudert WA, Ricordi C, Rodriquez-Rilo H, Behboo R, Finegold D, Trucco M: Evidence for superantigen involvement in insulin-dependent diabetes mellitus etiology. Nature 1994:**371**:351-355.

Faas SJ, Menon R, Braun ER, Rudert WA, Trucco M. Sequence-specific priming and exonuclease-released fluorescence detection of HLA-DQB1 alleles. Tissue Antigens 1996: **48**: 97-112.

Fakhrai-Rad H, Pourmand N, Ronaghi M. Pyrosequencing: an accurate detection platform for single nucleotide polymorphisms. Hum Mutat. 2002: **19**: 479-85.

Friday RP, Trucco M, Pietropaolo M. Genetics of Type 1 diabetes mellitus. Diabetes Nutr Metab. 1999: **12**: 3-26.

Gharizadeh B, Nordstrom T, Ahmadian A, Ronaghi M, Nyren P. Long-Read Pyrosequencing Using Pure 2'-Deoxyadenosine-5'-O'-(1-thiotriphosphate) Sp-Isomer. Anal Biochem. 2002: **301**: 82-90.

Gregory SG, Sekhon M, Schein J, Zhao S, Osoegawa K, Scott CE, Evans RS, Burridge PW, Cox TV, Fox CA, Hutton RD, Mullenger IR, Phillips KJ, Smith J, Stalker J, Threadgold GJ, Birney E, Wylie K, Chinwalla A, Wallis J, Hillier L, Carter J, Gaige T, Jaeger S, Kremitzki C, Layman D, Maas J, McGrane R, Mead K, Walker R, Jones S, Smith M, Asano J, Bosdet I, Chan S, Chittaranjan S, Chiu R, Fjell C, Fuhrmann D, Girn N, Gray C, Guin R, Hsiao L, Krzywinski M, Kutsche R, Lee SS, Mathewson C, McLeavy C, Messervier S, Ness S, Pandoh P, Prabhu AL, Saeedi P, Smailus D, Spence L, Stott J, Taylor S, Terpstra W, Tsai M, Vardy J, Wye N, Yang G, Shatsman S, Ayodeji B, Geer K, Tsegaye G, Shvartsbeyn A, Gebregeorgis E, Krol M, Russell D, Overton L, Malek JA, Holmes M, Heaney M, Shetty J, Feldblyum T, Nierman WC, Catanese JJ, Hubbard T, Waterston RH, Rogers J, de Jong PJ, Fraser CM, Marra M, McPherson JD, Bentley DR. A physical map of the mouse genome. Nature. 2002: **418**: 743-750.

Hsia S, Tong JY, Parris GL, Nghiem DD, Cottington EM, Rudert WA, Trucco M. Molecular compatibility and renal graft survival--the HLA DRB1 genotyping. Transplantation 1993: **55**: 395-399.

Huber SA, Graveline D, Born WK, O'Brien RL: Cytokine production by Vβ-T-cell subsets is an important factor determining $CD4^+$-Th-cell phenoytpe and susceptibility of BALB/c mice to Coxsackievirus B3-induced myocarditis. J Virol 2001:**75**:5860-5864.

Luppi P, Zanone MM, Hyoty H, Rudert WA, Haluszczak C, Alexander AM, Bertera S, Becker D, Trucco M: Restricted TCR Vβ gene expression and enterovirus infection in Type I diabetes: a pilot study. Diabetologia 2000: **43**:1484-1490.

Peakman M, Stevens EJ, Lohmann T, Narendran P, Dromey J, Alexander A, Tomlinson AJ, Trucco M, Gorga JC, Chicz RM. Naturally processed and presented epitopes of the islet cell autoantigen IA-2 eluted from HLA-DR4. J Clin Invest. 1999: **104**: 1449-1457.

Pourmand N, Elahi E, Davis RW, Ronaghi M. Multiplex Pyrosequencing. Nucleic Acids Res. 2002: **30**: e31.

Ringquist S, Alexander AM, Rudert WA, Styche A, Trucco M. Pyrosequence Based Typing of Alleles of the HLA-DQB1 Gene. Biotechniques 2002: **33**: 166-175.

Robinson J, Malik A, Parham P, Bodmer JG, Marsh SGE. IMGT/HLA database - a sequence database for the human major histocompatibility complex. Tissue Antigens 2000: **55**: 280-287.

Robinson J, Waller MJ, Parham P, Bodmer JG, Marsh SGE. IMGT/HLA Database - a sequence database for the human major histocompatibility complex. Nucleic Acids Research 2001: **29**: 210-213.

Ronaghi M. Pyrosequencing Sheds Light on DNA Sequencing. Genome Research 2001: **11**: 3-11.

Sollid LM. Coeliac disease: dissecting a complex inflammatory disorder. Nat Rev Immunol. 2002: **2**: 647-55.

Tong JY, Hsia S, Parris GL, Nghiem DD, Cottington EM, Rudert WA, Trucco M. Molecular compatibility and renal graft survival--the HLA DQB1 genotyping. Transplantation 1993: **55**: 390-395.

Trucco M. To be or not to be Asp 57, that is the question. Diabetes Care. 1992: **15**: 705-715.

KEY RESEARCH ACCOMPLISHMENTS

During the past 12 months of operation, we have been able to:

- Determine the antigenicity of CVB4 infected Vero cell lysates for specifically expanding T cells carrying Vβ7.1$^+$ TCR. The genes encoding the various capside proteins of CVB4 were cloned and the products will be tested independently or in meaningful combinations for T cell activation.

- Pyrosequence the genes encoding HLA-DQB1 and DRB alleles in homozygous and heterozygous cell lines, solving the problem of coamplification of alleles and pseudogenes.

- Set up the basis for using pyrosequencing in the search of SNPs associated with diabetes resistance in the mouse genome and SNPs associated with complications' susceptibility in the human genome.

REPORTABLE OUTCOMES / CONCLUDING REMARKS

The basis for high-throughput molecular HLA typing to better define susceptibility/resistance to type 1 diabetes has been defined and the laboratory set up for analyzing literally thousands of samples per week.

Also, the experience accumulated in sequencing HLA gene alleles was immediately used to set up a rational approach to scan the mouse and human genomes to define, respectively, other genes beside MHC that confer resistance to the disease in the mouse genome, and loci/genes that confer susceptibility to type 1 diabetes complications.